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OPTIMAL CONDITIONS IN THE STARCH-GEL ELECTROPHORESIS OF HEAT-DENATURED COLLAGEN

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ABSTRACT

The following details in the starch-gel electrophoresis of heat-denatured collagen in acetate buffer were considered: gel concentration, pH, ionic strength inside the gel and in the electrode compartments, voltage gradient, and duration of the run.

The recommended conditions are: outside temperature, +38°C.; concentration, 14.7 g.%; pH, 4.70–4.80; ionic strength inside the gel (critical) and in the buffer compartments, $\mu = 0.017$; voltage gradient, about 8.5–9 v/cm; and duration of the run, 3–4 hr.

A suitable arrangement for the gel support and the detailed procedure are described.

The inferences on the properties of the collagen subunits are discussed.

INTRODUCTION

During our work on the starch-gel electrophoresis of heat-denatured collagen (1) we observed that the electrophoretic pattern depended critically on the relevant details, and a systematic study of the conditions seemed justified. The results below show that the useful ranges are not only narrow but also interdependable and that for the reproduction of the patterns and for optimal resolution close adherence to specified conditions is necessary. The variation of the conditions affects the various subunits in different manner.

EXPERIMENTAL

Material.—In all the experiments the test material was heat-denatured (15 min. at 40°C.), purified, neutral-salt–soluble collagen (2) of guinea-pig skin, in 0.01*M* acetate buffer at pH 4.8. Runs were made at +38°C. in a temperature-controlled room.

Equipment.—The gel was supported in a plastic trough illustrated in Fig. 1. The gel was in contact with the buffer in the electrode compartments without any additional bridges. The thickness of the gel sheets could be adjusted in the range of 0–6 mm. The breadth of the gel troughs varied.

The construction of the electrode vessels was adopted from Kohn (3). The power supply was constructed in our workshop. Its capacity was 0–250 V., 0–100 mA. The voltage was kept constant by manual adjustments.

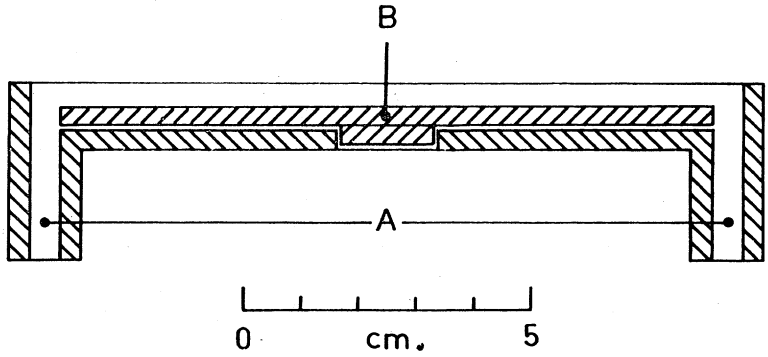

FIGURE 1.—Longitudinal section of the trough for starch-gel electrophoresis. Material is 3-mm.-thick transparent plastic. The whole unshaded volume is filled with starch gel. During the run the gel is covered with a thin plastic sheet. The gel-filled "feet" *A* dip into the buffer compartments functioning as bridges. After the run the horizontal gel sheet is lifted with a finger inserted through the hole in the center of *A*, where the appendage of *B* fits. The thickness of the gel can be adjusted by varying the thickness of *B*. If needed, the gel can be sliced horizontally in the same trough if suitable blocks are put between *A* and *B*. The standard size of the gel sheet was 110 x 48 x 6 mm.

Gel concentration.—Figure 2 shows that the migration of the fastest band is inversely proportional to the gel concentration, which is in agreement with the findings of Smithies on other proteins (4). Choosing a suitable pH and ionic strength it was possible to achieve good resolution within the range of gel concentrations used (9.70–16.35 g.%). In low gel concentrations the bands were slightly blurred, presumably because of diffusion.

The range of 14–15 g.% was a suitable compromise for the gel concentration. The bands were sharp, the useful pH range was wider than at lower gel concentrations, the duration of the run was kept within practical limits by the available voltages, and at this concentration the gel was not yet too viscous to be cast. These arguments hold at the temperature +38°–55°C. inside the gel during the run.

Hydrogen ion concentration.—In our original method we used acetate buffer, but to expand the pH range a few experiments were made with Michaelis' buffer (Fig. 3). The useful resolution was obtained only in the range of pH 4–5,

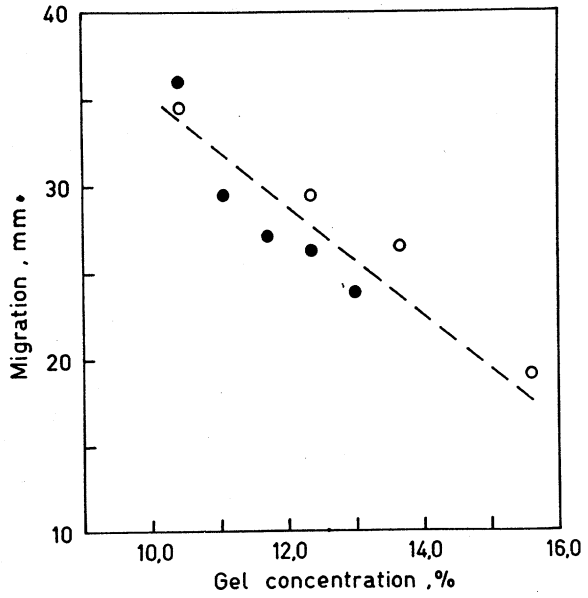

FIGURE 2.—Effect of gel concentration on the migration of the most rapid band. Conditions: pH, 4.47; ionic strength inside the gel, $\mu = 0.017$; voltage, 100v; duration, 3.5 hr. Symbols indicate the two experimental series.

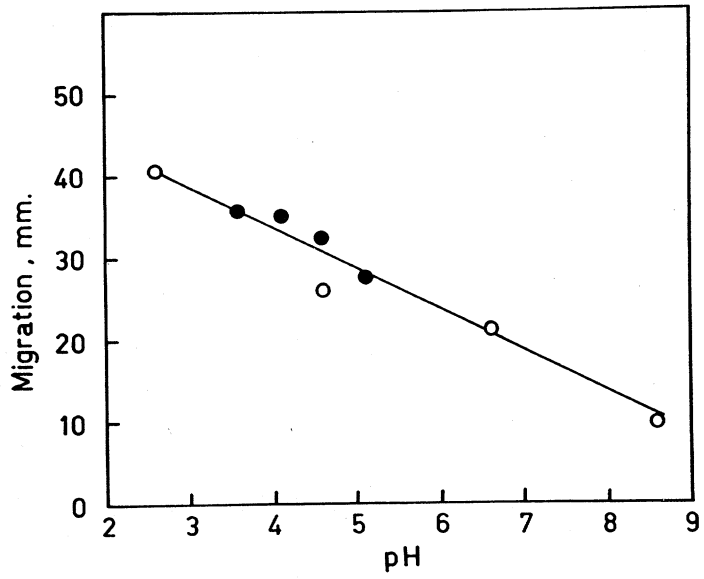

FIGURE 3.—Effect of pH on the migration of the most rapid band ($\alpha 2$). ● acetate buffer; ○ Michaelis' buffer. Conditions: gel concentration, 13.0 g.%; ionic strength inside the gel, $\mu = 0.022$; voltage gradient, 5.8 v/cm.; duration, 4 hr.

which is demonstrated in Fig. 4. The optimal pH was searched at different gel concentrations, and it was observed that at lower gel concentrations the op-

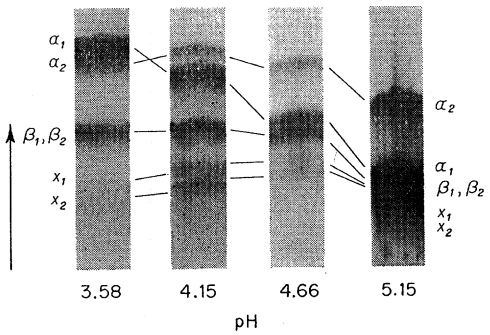

FIGURE 4.—Effect of pH (range 3.58–5.15) on the resolution of the fractions from heated neutral-salt–soluble collagen. Conditions: gel concentration, 13.0 g.%; ionic strength inside the gel, $\mu = 0.022$; acetate buffer; voltage gradient, 5.8 v/cm.; duration 4 hr.

timal resolution is achieved at lower pH levels than those preferred with more condensed gels. When we choose the gel concentration of 14.7 g.%, the corresponding optimal pH range is of special interest (Fig. 5). The best patterns at

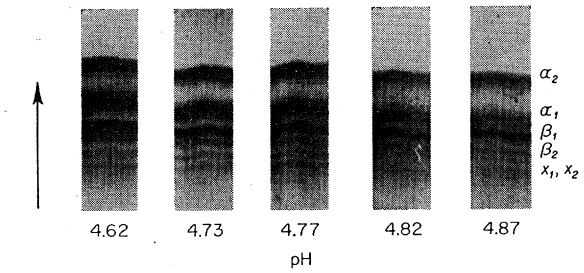

FIGURE 5.—Effect of pH (range 4.62–4.87) on the resolution of fractions from heated neutral-salt–soluble collagen. Conditions: gel concentration, 14.7 g.%; ionic strength inside the gel, $\mu = 0.022$; acetate buffer; voltage gradient, 5.8 v/cm; duration, 3.5 hr.

this gel concentration were obtained at pH 4.73–4.87. We note that the pattern at pH 4.62 differs appreciably from the pattern at pH 4.87. The following observations on the behavior of different subunits may be added: Fractions $\beta 1$ ($= \beta_{12}$)* and $\beta 2$ ($= \beta_{11}$)* separated from each other at pH 4.40 only when gel concentrations were less than about 12 g.%, but at higher pH their resolution was obtained also at higher gel concentrations. The relative migration of $\alpha 1$ (in reference to $\alpha 2$) decreased with increasing pH and with increasing gel

*In the parentheses are given the new designations of $\beta 1$ and $\beta 2$, as suggested recently. In the figures β_1 means $\beta 1$, etc.

concentrations. Especially the $x1$ and $x2$ were retarded in high gel concentrations but only at rather low pH.

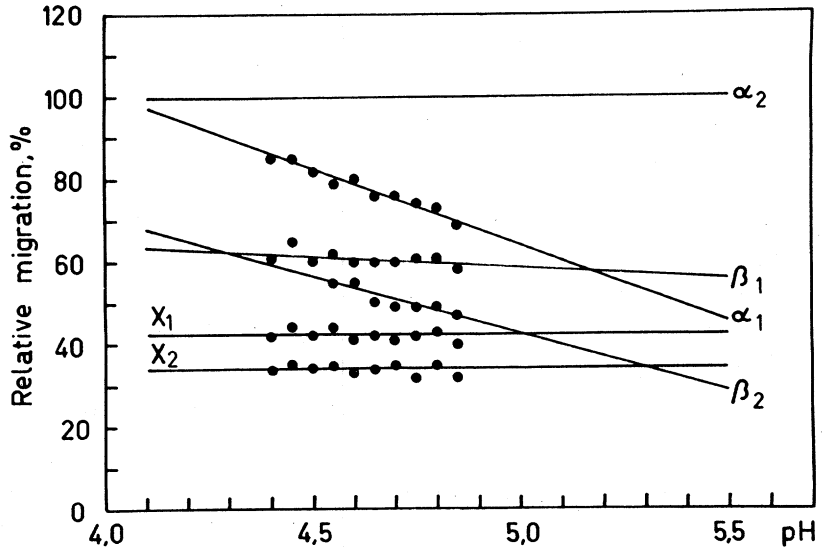

FIGURE 6.—Effect of pH on the relative migration (in reference to the most rapid band $\alpha 2$) of various subunits from heated neutral-salt-soluble collagen.

Further work is in progress on the fractions, which were tentatively designated as x_1 and x_2. When their mobilities were plotted against the reciprocals of gel concentrations, as suggested by Smithies (4), they seemed to be larger aggregates than the β-components.

Figure 6 illustrates the relative migration rates of different subunits. It is obvious that the electrophoretic pattern varies with the pH selected. From the relative migration rates it is observed that $\alpha 1$ and $\beta 2$ ($= \beta_{11} = \alpha 1 - \alpha 1$) belong to a different group than the others: $\alpha 2$, $\beta 1$ ($= \beta_{12} = \alpha 1 - \alpha 2$), $x1$, and $x2$. The components containing $\alpha 1$ only seem to have an isoelectric point more acid than the rest.

The ionic strength.—At first we followed the instructions of most other workers (5) and used in the electrode compartments buffers of higher ionic strength compared to the buffer inside the gel. Later we found that the salt concentration in the electrode compartments was not important in this arrangement. If the ionic strength inside the gel was constant, the only change caused by increasing the ionic strength in the electrode compartments was an augmented migration and gel temperature because of increased current. No change was observed in the resolution or in the mutual relationship between the different bands.

With conductivity measurements it was confirmed that the ionic strength inside the gel was to a certain extent independent of the salt concentration in electrode compartments.

The most critical factor for a good resolution was the ionic strength inside the gel, i.e., in the buffer which was used in the preparation of the gel. Figure 7

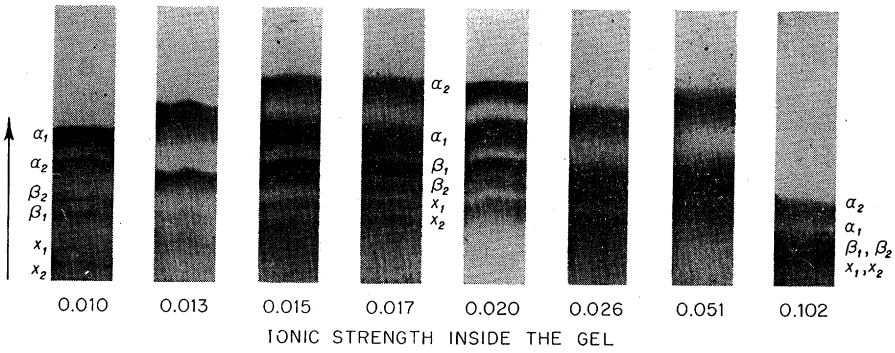

FIGURE 7.—Effect of the ionic strength inside the gel on the resolution of the fractions from heated neutral-salt–soluble collagen. Conditions: gel concentration, 14.7 g.%; pH, 4.77; acetate buffer; voltage gradient, 5.8 v/cm; duration, 3.5 hr.

shows the effect of the ionic strength. Below, in the paragraph of the recommended procedure, we give the composition of the acetate buffer of $\mu = 0.017$.

Voltage gradient and gel temperature.—When a voltage of 150 V. is applied to the electrodes, we get a gradient of about 8.7 v/cm. The temperature rises to +55°C. inside the gel, and the current increases from 24 to 34 mA during a run of 2.5 hr. (ionic strength $\mu = 0.017$). When higher voltages were tried, the final temperature increased so much that the viscosity of the gel decreased. At voltages of 200 V. or more the bands became hazy and blurred, because of diffusion and because of the possible continued destruction of collagen fractions which happens during heating over 24 hr. even at +40°C. (6). There was thus no advantage in increased voltage. No attempts were made to stabilize the temperature inside the gel.

Duration.—In some experiments the duration was extended from 3–4 hr. to 6 hr. The migrated distances increased proportionally, and a good resolution was maintained but not improved.

RECOMMENDED PROCEDURE

Buffer.—The pH 4.7 acetate buffer of $\mu = 0.017$ is made as follows:

Stock solutions:

A. 1.5*M* acetic acid contains in 1000 ml. of solution 90.0 g. or 85.8 ml. of glacial acetic acid, *p.a., sp.gr.* 1.05.

B. 1.5*M* sodium acetate solution contains in 1000 ml. of solution 123.06 g. of anhydrous sodium acetate, *p.a.,* or 204.1 g. of sodium acetate x $3H_2O$, *p.a.*

For use 9.33 ml. of solution *A* and 11.33 ml. of solution *B* are mixed and diluted to 1000 ml.

Preparation of the gel.—Eleven grams of hydrolyzed starch (Connaught Medical Laboratories Ltd., Toronto, Canada) is suspended into 75 ml. of the final buffer to get a gel of 14.7 g.%. The suspension is heated in a 500-ml. Erlenmeyer flask under continuous stirring on an open flame. The suspension turns transparent in about one minute, and the heating is continued until the gel becomes fluid (in about 30 sec.). The gel is deaerated by evacuation with a water pump about 30 sec. after the gel begins to boil (this step may be omitted). The jelly is then poured into the trough, which is carefully covered with a glass plate, avoiding the formation of air bubbles, and the gel sheet is allowed to cool at room temperature for at least 2 hr. After about 30 min. at +38°C. the gel is ready for electrophoresis.

Preparation of the collagen sample.—The sample is denatured at +40°C. for 15 min. immediately before the electrophoresis.

Application of the sample.—The solution of denatured collagen, containing about 0.3–0.5 mg. of protein, is imbibed into a 4 x 10 mm. piece of Whatman No. 3 MM filter paper. The paper should not be allowed to dry, otherwise the resolution is impaired. A perpendicular slot is made with a razor blade in the anode end across the gel, and the moist paper with the sample is inserted. Two or three samples can be run simultaneously in the 48-mm.-broad gel, which is covered with a thin plastic sheet during the run.

Staining of the gel.—The gel can be sliced horizontally, if needed, in the trough with a cutting wire as explained in the legend of Fig. 1.

To stain the gel we have adopted the method described by Vahvaselkä (7). The gel is heated at +90°C. about 15 min. in a solution of 0.1% water-soluble nigrosine (*Fluka AG.* 1, Buchs, *Switzerland*) in 50% (v/v) aqueous glycerol. After the staining period the gel is washed in 50% glycerol with a gentle continuous agitation. This procedure gives transparent, durable, and flexible gels, which are suitable for densitometry as they are or after "plastification" according to Baur (8).

DISCUSSION

The most critical details for the optimal resolution were the ionic strength and pH. It seems relevant that the isoelectric point of collagen depends on the ionic strength of the environment (9), so that with increasing ionic strength the isoelectric point shifts to a lower pH. This finding is in agreement with our results,

i.e., if the ionic strength is increased from the optimal, the effect is the same as changing the environmental pH to alkaline direction. The importance of electrolyte composition and concentrations on the properties of collagen macromolecules has been emphasized also by Kahn *et al.* (10) and is also evidenced in the fractionation of collagen subunits by elution of ion-exchange materials with salt strength gradients (11–14).

The subunits are not equally sensitive to these conditions, and the patterns, therefore, change when the ionic strength is altered. The sequence of the bands may be reversed, and the composition of the apparent bands varies. For example, at low ionic strength or at pH <4.0 $\alpha 1$ can migrate more rapidly than $\alpha 2$, or $\beta 2$ $(= \beta_{11})$ may pass $\beta 1$ $(= \beta_{12})$ as shown in Fig. 6. Analogously other combinations are formed when the ionic strength or pH are raised. The subunits may have different isoelectric points. Judging from the analyses of Piez *et. al.* (11), the $\alpha 1$-unit of rat-skin and rat-tail-tendon collagen contains more acid and less basic groups than the $\alpha 2$-unit.

The variation in the gel concentrations does not affect the electrophoretic behavior of the α- and β-units similarly because of the sieve effect. We do not have any obvious explanation for the interdependence of gel concentration and optimal pH range. Starch contains some carboxyl groups, which may modify the migrations.

If the temperature rose above about $+55°$C., the bands became blurred, and the question arises, as to the safe upper limit of temperature in the gel, which also limits the current and voltage gradient. We cannot be positive on this point but only state that the patterns with final temperature in the gel about $+55°$C. were not appreciably different from those where the final temperature was only 45–50°C. because of a lower voltage gradient. After long heating of collagen solution before electrophoresis at $+40°$C. (6) there are qualitative changes in the electrophoretic pattern which were not observed after the runs of only 3–4 hr. with previous heating for 15 min.

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REFERENCES

1. Näntö, V., Maatela, J., and Kulonen, E. *Acta Chem. Scand.*, **17**, 1604 (1963).
2. Gross, J. *J. Exp. Med.*, **107**, 247 (1958).
3. Kohn, J. *Clin. Chim. Acta*, **2**, 297 (1957).
4. Smithies, O. *Arch. Biochem. Biophysics*, **suppl. 1**, 125 (1962).
5. ———. *Adv. Protein Chem.*, **14**, 65 (1959).

6. Hollmén, T., and Kulonen, E. *Acta Chem. Scand.,* **18**, 1027 (1964).
7. Vahvaselkä, E. *Nature,* **193**, 474 (1962).
8. Baur, E. W. *J. Lab. Clin. Med.,* **61**, 166 (1963).
9. Jackson, D. S., and Neuberger, A. *Biochim. Biophys. Acta,* **26**, 638 (1957).
10. Kahn, L. D., Carroll, R. J., and Witnauer, L. P. *Ibid.,* **63**, 243 (1962).
11. Piez, K. A., Eigner, E. A., and Lewis, M. S. *Biochemistry,* **2**, 58 (1963).
12. ———, Weiss, E., and Lewis, M. S. *J. Biol. Chem.,* **235**, 1987 (1960).
13. Kulonen, E., Virtanen, U. K., and Salmenperä, A. *Acta Chem. Scand.,* **16**, 1579 (1962).
14. Candlish, J. K. *Biochim. Biophys. Acta,* **74**, 275 (1963).

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